

Determination of amino acids of plants from *Angelica* L. genus by HPLC method

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Abstract

One of the tasks of pharmaceutical science is to find new sources of effective drugs. Such sources include plants such as *Angelica archangelica* L. and *Angelica sylvestris* L., which have been used for many years to treat various diseases in folk medicine. Because the chemical composition of these plants is poorly understood, the aim of our study was to investigate the amino acid composition of the leaves of *A. archangelica* L. and *A. sylvestris* L. The amino acids of the leaves of the study species of the genus *Angelica* L. were determined by the HPLC method. Eighteen free and nineteen bound amino acids were identified in the leaves of *A. archangelica* L. The *A. sylvestris* L. leaves contained nineteen free and the same amount of bound amino acids. High concentrations of free and bound amino acids such as L-glutamic acid and L-aspartic acid predominate in *A. archangelica* L. and *A. sylvestris* L. This allowed these amino acids to be considered distinguishing markers of the study plants. Character metabolic processes in which these amino acids take part may be associated with the medicinal properties of these plants pursuant to their use in medicine and, therefore, may contribute to the insight of their therapeutic properties.

Keywords

Angelica archangelica L., *Angelica sylvestris* L., leaves, amino acids, HPLC

Introduction

One of the tasks of pharmaceutical science, at the present stage of development, is to find new sources of effective drugs (Feshchenko et al. 2021a). This is possible due to the expansion of the range of medicinal plants through the full use of own resources of wild raw materials. When creating new herbal remedies, scientists pay special attention to plants that have long been used in folk medicine (Budniak et al. 2021a, b, e; Marchyshyn et al. 2021c; Vasenda et al. 2021). The advantage of herbal medicine is the availability and relative cheapness compared to synthetic drugs. Herbal medicines show a wide range of pharmacological properties, which are realized through different groups of biologically active substances (Darzuli

et al. 2021; Slobodianiuk et al. 2021d). Herbal medicines are low-toxic, can be used for a long time, have fewer side effects on the human body, and are well combined with synthetic drugs (Stoiko and Kurylo 2018; Darzuli et al. 2019; Budniak et al. 2020; Huzio et al. 2020; Budniak and Vasenda 2021). Plants of the genus *Angelica* L. are promising in this regard.

Angelica L. is among the largest genus of the *Apiaceae* family. It has approximately 110 species of biennial and perennial herbs (Daşkın and Kaynak 2012; Liao et al. 2020). More than 50 of these species are used in traditional medicine in many countries (Lobiuc et al. 2012; Ağalar et al. 2020). *Angelica* species are found on all continents of the Northern Hemisphere, reaching as far north as Iceland, Lapland, Greenland, and East Asia host the highest

number of species (Liao et al. 2012; Pimenov 2017; Wang et al. 2017).

For many centuries, species of this genus namely *A. archangelica*, *A. sylvestris*, *A. californica*, *A. atropurpurea*, *A. dahurica*, *A. keiskei*, *A. lucida*, *A. tomentosa*, *A. venenosa*, *A. tenuissima*, *A. kingie* have been used as an expectorant, diuretic, anti-inflammatory, and diaphoretic, and means for flu, chronic bronch, pleurisy, colds, fever, typhoid, coughs, hepatitis, indigestion, colic, headaches, rheumatism, arthritis, bacterial and fungal infections (Sarker and Nahar 2004). These properties are the result of the availability of many groups of biologically active compounds. Among them are coumarins, sesquiterpenes, flavonoids, polysaccharides, acetylenic compounds, and chalcones (Skakun and Stepanova 1988; Sarker and Nahar 2004; Wang et al. 2012; Wang et al. 2017; Shawky and Abou El Kheir 2018; Xie et al. 2018; Karakaya et al. 2020; Alkan Türkuçar et al. 2021).

A. archangelica and *A. sylvestris* are the most common species in Ukraine. They grow on wetlands and waterfronts (Ağalar et al. 2020). *A. archangelica* has a wide range of biological activities namely anti-inflammatory, antispasmodic, diuretic, antimutagenic, diaphoretic, sedative, cytotoxic, and anxiolytic properties, increases bile secretion, secretion of gastric and pancreatic juice, enhances intestinal motor function, inhibits fermentation (Salikhova and Poroshenko 1995; Pathak et al. 2010). The root is used for bronchitis, gastric ulcers, anorexia, chronic fatigue, migraine, and obstetric complaints (Bhat et al. 2011; Maurya et al. 2017). Yeh et al. established that *A. archangelica* is a cytoprotective agent effective against chronic hepatotoxicity, which hence indirectly protects the liver from oxidative stress (Yeh et al. 2003). Khayyal et al. found that extract of *A. archangelica* has anti-ulcerogenic activity associated with an increase in prostaglandin E2 release, a reduced acid output, a decrease in leukotrienes, and increased mucin secretion (Khayyal et al. 2001). Sigurdsson et al. revealed that the antiproliferative activity of a tincture is from fruits of this plant. It is results presence of *A. archangelica* two furanocoumarins, such as xanthotoxin (8-methoxy-psoralen) and imperatorin (8-isopentenyl-oxypsoralen) (Sigurdsson et al. 2004; Sigurdsson and Gudbjarnason 2007; Wszelaki et al. 2011). Also, antitumor activity in vivo and antiproliferative activity in vitro of extract of *Angelica archangelica* leaves were found by researchers (Sigurdsson et al. 2005). The essential oil obtained from *A. archangelica* roots has good antimicrobial activity against *Enterococcus faecalis*, *Peptostreptococcus anaerobius*, *Clostridium perfringens*, *Clostridium difficile*, *Eubacterium limosum*, and *Candida albicans*. The main proportion of oil consists of monoterpene (up to 88%). α -pinene (21.3%), δ -3-carene (16.5%), limonene (16.4%), and α -phellandrene (8.7%) are its major compounds (Chalchat and Garry 1997; Doneanu and Anitescu 1998; Nivinskiene et al. 2003; Fraternali et al. 2014; Simonović et al. 2014). Also, *A. archangelica* contains the following biologically active substances: bittering agents, flavonoids, tanning agents,

resins, silica, carbohydrates, terpenes, and organic acids (Wahlin and Blixt 1994).

A. archangelica is a plant that is part of the European Pharmacopoeia, the Ukrainian Pharmacopoeia, the British Herbal Pharmacopoeia, the Complete German Commission E, the British Pharmacopoeia, and many other pharmacopoeias.

A. sylvestris, also known as “European wild angelica”, “wild angelica and is analogous to *A. archangelica* in terms of their common characteristics. As a result of ethnobotanical research, the use was established *A. sylvestris* disorders of digestive, respiratory, and nervous systems, fever, infection and influenza, in cases of indigestion, as an analgesic, anticarcinogenic, antipyretic remedy (Özek et al. 2008; Vogl et al. 2013). *A. sylvestris* contain simple coumarins and furanocoumarins, such as umbelliprenin, aviprin, imperatorin, bisabolangelone, bergapten, xanthotoxin, isoimperatorin, byakangelicin, 5- β -cyclohexandulyloxy-psoralen, 1'-O- β -D-glucopyranosyl-(2S,3R)-3-hydroxymarmesin, 4-[(2,4,4-trimethyl-1-cyclohexen-1-yl)methoxy]-7H-furo[3,2-g][1]-benzopyran-7-one, etc (Muckensturm et al. 1981; Carbonnier and Molho 1982; Lemmich et al. 1983; Murphy et al. 2004). Sitosterol, campesterol, a-saccharostenon, stigmaterol, ergosta-5,24-dien-3 α -ol, cholesterol, stigmast-7-en-3-ol were defined in the plant (Krzaczek and Nowak 2000). Ağalar et al. determined that the main compounds of essential oil of *A. sylvestris* were α -pinen (42.0%) and β -phellandrene (25.5%) in the flower; 10-epi- γ -eudesmol (5.4%), elemol (5.4%), spathulenol (4.8%) in the root; spathulenol (12.4%), germacrene D (10.6%), α -humulene (7.6%) in the leaves; α -pinen (23.2%) and β -phellandrene (9.1%) in the fruit (Ağalar et al. 2020).

Previous studies revealed various groups of biologically active substances in *A. archangelica* and *A. sylvestris*, but amino acids were not studied. Based on the literature data and analysis of the chemical composition of biologically active substances of *A. archangelica* and *A. sylvestris*, it is important to study the amino acids composition of these plants.

Material and method

Plant materials

The leaves of *A. archangelica* and *A. sylvestris* were collected in Ukraine, on meadows and swamps of the Ternopil region during the flowering period in July-August 2017. The leaves of the study objects were authenticated by professor Svitlana Marchyshyn (TNMU, Ternopil, Ukraine) (Budniak et al. 2021c, d). A voucher specimen was deposited in the laboratory herbarium of the Department of Pharmacognosy and Medical Botany (TNMU, Ternopil, Ukraine) (Marchyshyn et al. 2021b, d; Slobodianiuk et al. 2021a, c). The study raw materials were then dried, crushed, and stored according to the general GACP requirements (WHO 2003; Husak et al. 2018).

Standards and chemicals

Standards of amino acids, including L-asparagine (Asn), L-glutamic acid (Glu), L-alanine (Ala), L-leucine (Leu), L-serine (Ser), L-isoleucine (Ile), L-aspartic acid (Asp), L-arginine (Arg), Glycine (Gly), L-valine (Val), L-methionine (Met), L-cystine (CyS-SCy), L-cysteine (Cys), L-phenylalanine (Phe), L-threonine (Thr), L-glutamine (Gln), L-proline (Pro), L-histidine (His), L-tyrosine (Tyr), L-lysine (Lys) obtained from Sigma (Sigma-Aldrich, St. Louis, MO, USA), were of analytical grade (> 99% purity) (Slobodianiuk et al. 2021b, e).

Reagents 9-fluorenylmethyl chloroformate (FMOC-Cl), Hydrochloric acid (HCl), Acetonitrile (ACN), β -mercaptoethanol, Amantadine hydrochloride were from Sigma-Aldrich (USA). All other reagents were of the highest purity. The water used in the studies was produced by the MilliQ Gradient water deionization system (Millipore, USA).

Chromatographic equipment

The amino acids composition of the leaves of *A. archangelica* and *A. sylvestris* is determined by the HPLC method on high-performance liquid chromatograph Agilent 1100 (Agilent Technologies, USA) equipped with a flow-through vacuum degasser G1379A, a four-channel pump of the low-pressure gradient G13111A, an automatic injector G1313A, a column thermostat G13116A, and a diode array detector G1316A. Samples were analyzed using a column length ZORBAX-XDB-C18 - 50 mm, inner diameter - 4.6 mm, and the diameter of octadecylsilyl sorbent grain 1.8 μ m.

Sample preparation for the analysis of plant raw materials

For the extraction of free amino acids the samples of the study raw material were ground into a powder by laboratory mill, then about 0.3 g (accurately weighed) was selected and placed into a flask with 3.0 ml of 0.1 M aqueous solution of hydrochloric acid containing 0.2% β -mercaptoethanol. The extractions were carried out in the ultrasonic water bath at 50 °C for 2 hours.

Extraction of bound amino acids was carried out by adding 3 ml of 6 M an aqueous solution of hydrochloric acid containing 0.4% β -mercaptoethanol to 0.2 g (accurately weighed) of powdered herbal raw materials. Hydrolysis was carried out for 24 hours in a thermostat at 110 °C.

The resulting extracts were centrifuged at 3000 rpm and filtered. Obtaining filtrates were placed in a vacuum desiccator at a temperature of 40–45 °C and a pressure of 1.5 mm Hg until hydrochloric acid is completely removed.

Then 200 μ l of 0.8 M borate buffer pH 9.0 and 200 μ l of a 20 mM solution of 9-fluorenylmethoxycarbonyl chloride (FMOC-Cl) in acetonitrile was sequentially added with an automatic dispenser. After a 10-minute, 20 μ l of a 150 mM solution of amantadine hydrochloride in 50% aqueous acetonitrile was added.

Chromatography conditions

For analysis, the following chromatography mode was set:

- gradient chromatography mode (Table 1);
- eluent working pressure 220–275 kPa; column thermostat temperature 50 °C;
- sample volume 2 μ l; The detection parameters were set as follows: measurement scale 1.0; scan time 0.5 s. The detection wavelength is 265 nm.

Table 1. Gradient chromatography mode.

Retention time, min	A% 0.05M sodium acetate solution, pH 6.5	B% 0.10 M sodium acetate solution: ACN = (23:22, v/v), pH 6.5	C% H ₂ O	D% ACN	mobile phase feed rate, mL/min
0	70	30	0	0	1.5
3.87	27	73	0	0	1.5
5.73	0	100	0	0	1.5
7.83	0	100	0	0	1.5
8.17	0	0	15	85	1.5
10.00	0	0	2	98	2.0
10.10	70	30	0	0	2.0
11.00	70	30	0	0	2.0

Identification and calculation of content of amino acids

The identification of amino acids was performed according to their hold-up time (using standards as a reference). The quantitative content of amino acids is calculated from the value of the peak area of the amino acids. The content of bound amino acids was determined by subtracting the content of free amino acids from their total content (Jámbor and Molnár-Perl 2009a, b; Chen et. al. 2010; Feshchenko et. al. 2021b).

Validation of the method

The validation method and the analysis procedure of the amino acid content were performed according to validation guides for EURACHEM analytical methods.

To evaluate the sensitivity and linearity of the signal in relation to the concentration, linear calibrations were generated for each amino acid. To determine the linearity of the amino acids, five different concentrations, namely 0.1 mg/100 g, 0.3 mg/100 g, 0.6 mg/100 g, 1.3 mg/100 g and 3.3 mg/100 g of standards were used for the amino acids. The correlation coefficients (R^2) ranged from 0.9981 to 0.9999, as shown in Table 2. The limit of detection (LOD) and limit of quantification (LOQ) of each analyte were determined as the concentration of a standard solution with $S/N = 3$ (signal-to-noise ratio) and $S/N = 10$ (Slobodianiuk et al. 2022). In Table 1, the LODs were calculated by dividing three times the standard error of the calibration line at the intercept by the slope of the calibration line, and the LOQs were calculated by dividing 10 times the standard error of the calibration line at the intercept by the slope of the calibration line. As tabulated in Table 1 the LODs and the LOQs were in the ranges of 0.0072–0.0593 mg/100g

and 0.0236–0.1977 mg/100g, respectively, depending on the amino acid under consideration. The performance parameters of the reference amino acid method, concentrations, limit of detection (LOD) and limit of quantification (LOQ) were statistically calculated using Statistica v 10.0 (StatSoft Inc.) (Marchyshyn et al. 2021a).

Table 2. Performance parameters of the amino acid determination method.

Amino acid	Correlation coefficient R ²	Limit of detection LOD, mg/100 g	Limit of quantification LOQ, mg/100 g
Asp	0.9999	0.0391	0.1307
Glu	0.9997	0.0138	0.0456
Asn	0.9981	0.0221	0.0744
Gln	0.9991	0.0168	0.0556
Ser	0.9989	0.0153	0.0508
Arg	0.9998	0.0184	0.0610
Gly	0.9994	0.0163	0.0551
Thr	0.9996	0.0335	0.1114
Ala	0.9991	0.0109	0.0360
Pro	0.9998	0.0186	0.0612
Val	0.9999	0.0524	0.1751
Met	0.9996	0.0149	0.0504
Ile	0.9999	0.0593	0.1977
Leu	0.9989	0.0112	0.0379
Phe	0.9995	0.0226	0.0749
CyS-SCy	0.9987	0.0181	0.0607
His	0.9993	0.0072	0.0236
Lys	0.9999	0.0432	0.1435
Cys	0.9987	0.0134	0.0452
Tyr	0.9996	0.0172	0.0579

Results and discussion

The amino acids profiles of *A. archangelica* leaves and *A. sylvestris* leaves were determined using HPLC method (Figs 1–4, Table 3).

Table 3. The amino acid composition content of the leaves of *Angelica archangelica* L. and *Angelica sylvestris* L.

Name of amino acid	Amino acids content, mg/100 g			
	<i>Angelica archangelica</i> L.		<i>Angelica sylvestris</i> L.	
	Free	Bound	Free	Bound
Asp	37.1±0.29	1298.5±12.86	66.9±0.47	861.0±8.51
Glu	130.9±1.47	1471.3±11.62	96.3±0.94	1155.6±19.67
Asn	8.1±0.02	2.8±0.03	377.6±2.19	5.4±0.02
Gln	15.8±0.19	n/d	547.8±7.26	n/d
Ser	18.7±0.26	779.2±5.81	90.4±0.72	294.3±2.95
Arg	14.3±0.40	735.2±6.49	22.0±0.19	269.1±3.67
Gly	19.1±0.34	830.1±6.34	14.6±0.11	363.1±7.25
Thr	15.8±0.19	660.8±5.98	82.9±1.75	258.4±2.18
Ala	14.6±0.17	849.1±6.42	25.6±0.21	320.4±4.22
Pro	25.0±0.24	667.2±6.13	148.1±1.37	235.4±5.31
Val	5.5±0.01	442.3±4.87	92.1±0.98	150.9±2.89
Met	1.1±0.02	95.2±0.83	15.4±0.18	17.7±0.36
Ile	2.9±0.04	334.1±1.78	51.0±0.11	127.9±1.97
Leu	5.4±0.11	766.9±5.12	34.9±0.65	286.7±5.13
Phe	4.8±0.09	458.1±4.64	38.1±0.27	187.4±3.78
CyS-SCy	n/d	6.7±0.05	n/d	6.0±0.14
His	7.1±0.07	238.7±1.08	16.0±0.41	126.0±2.49
Lys	10.4±0.13	435.2±1.13	21.1±0.37	214.3±3.76
Cys	n/d	20.9±0.16	0.4±0.01	8.4±0.03
Tyr	9.3±0.18	423.1±1.94	54.9±0.58	197.6±2.52

Note: n/d – not detected.

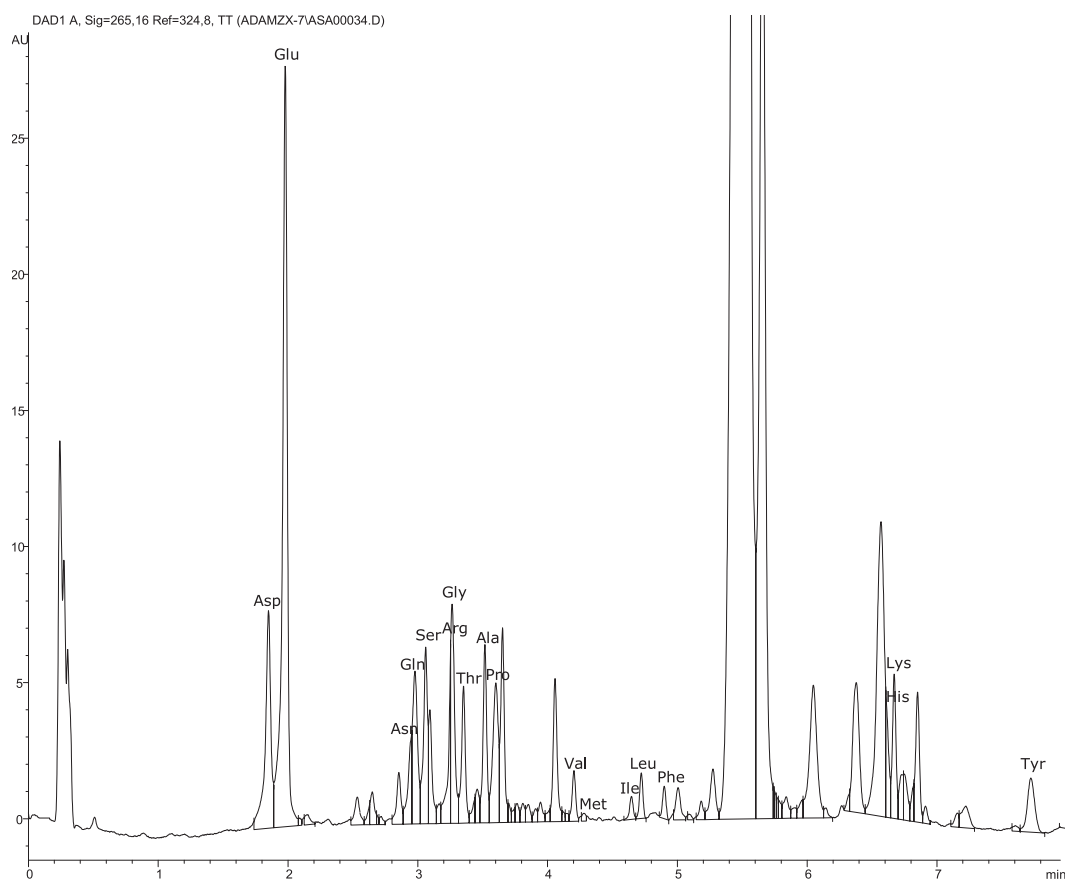


Figure 1. HPLC chromatogram of free amino acids of *Angelica archangelica* L. leaves.

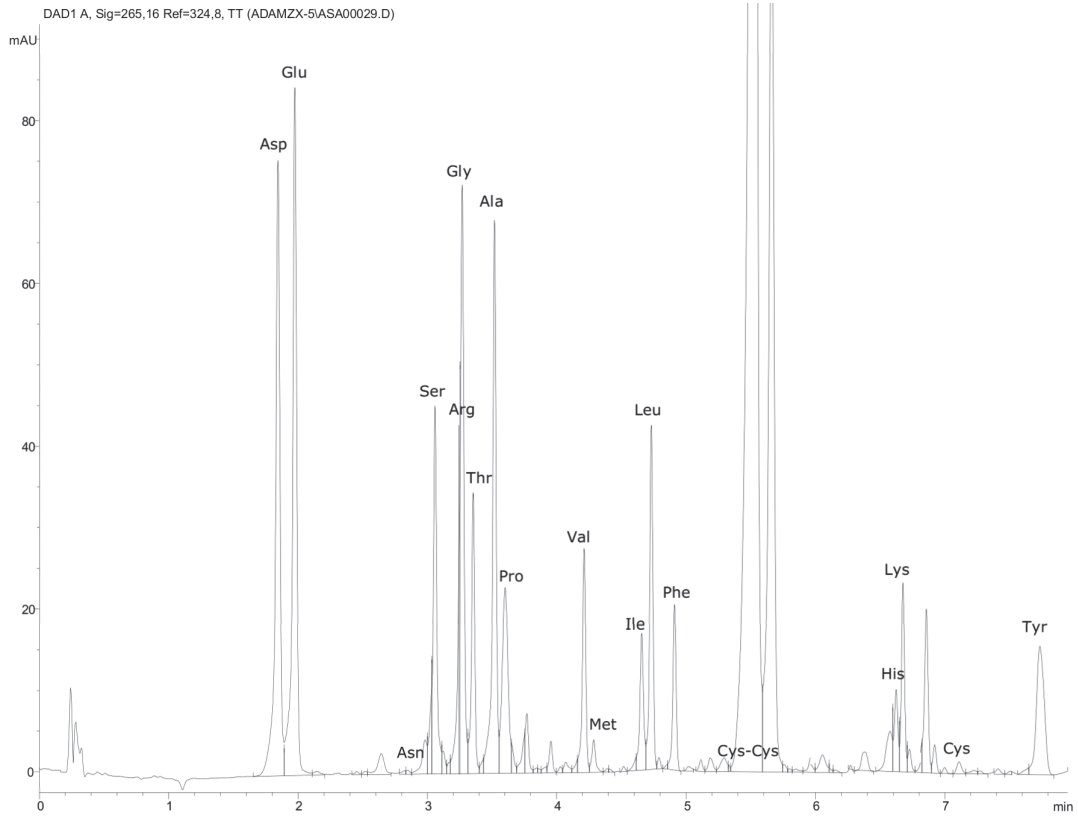


Figure 2. HPLC chromatogram of amino acids after hydrolysis of *Angelica archangelica* L. leaves.

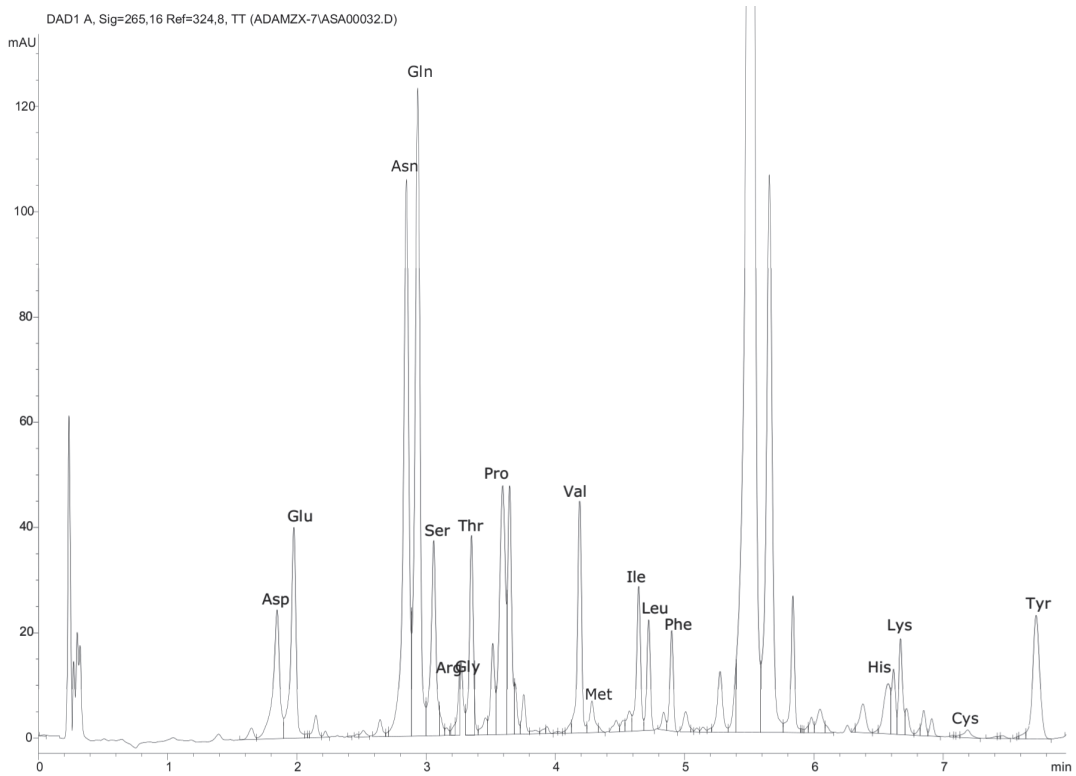


Figure 3. HPLC chromatogram of free amino acids of *Angelica sylvestris* L. leaves.

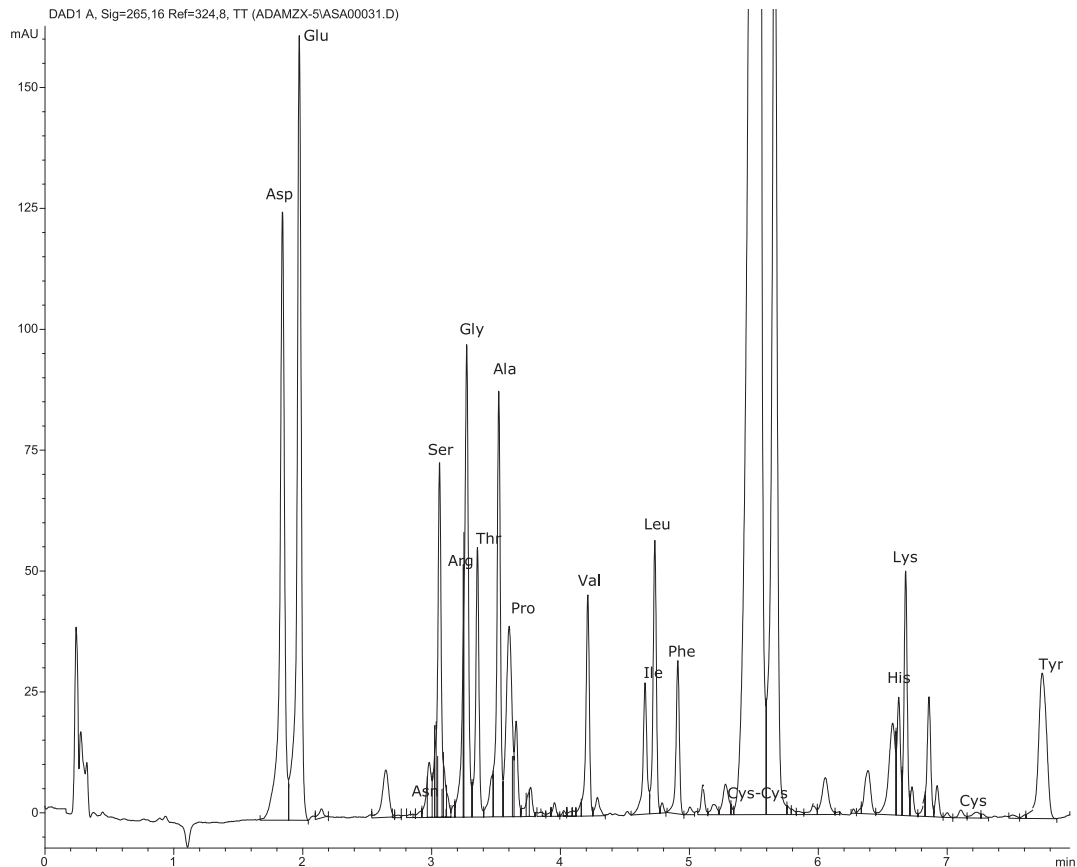


Figure 4. HPLC chromatogram of amino acids after hydrolysis of *Angelica sylvestris* L. leaves.

The HPLC method identified eighteen free amino acids in the leaves of *A. archangelica*. Among them, eight amino acids are essential namely L-threonine, L-valine, L-methionine, L-isoleucine, L-leucine, L-phenylalanine, L-histidine, and L-lysine. Two acids, such as L-arginine and L-tyrosine are semi-essential amino acids. The other eight amino acids are nonessential amino acids.

Free glutamic acid was present in the leaves of *A. archangelica* in the greatest amount, its content was 130.9 mg/100g. This acid takes part in a significant amount of metabolic reactions and affects metabolic processes in the brain. Glutamic acid is a source of glucose and holds its normal blood level (Okasha et al. 2019). It maintains acid-base homeostasis in blood and tissues (Lysisikov 2012). This acid is a precursor in the synthesis of ornithine and proline and contributes to the temporary neutralization of ammonia with the formation of non-toxic glutamine (Pharmaceutical encyclopedia 2016). Glutamic acid is used as a conjugate because it improves the efficiency of anticancer agent and reduces its toxicity in relation to normal cells. The synthetic amides acid show activity with relation to Ehrlich ascites carcinoma (Dutta et al. 2013). The content of other amino acids was lower.

Nineteen bound amino acids were identified in the leaves of *A. archangelica*. Eight amino acids are essential, eight – nonessential, and three acids namely L-arginine, L-cysteine, and L-tyrosine are semi-essential ami-

no acids. Among the bound amino acids, L-glutamic and L-aspartic acids were present in the leaves of *A. archangelica* in the greatest amount, their content was 1471.3 mg/100g, 1298.5 mg/100 g, respectively. Aspartic acid is a major component of antibodies, immunoglobulins, and the immune system as a whole. With this acid, carbohydrates are transformed into energy which helps the body to reduce ammonia levels after exercise. It has hepatoprotective properties and is involved in reamination reactions, and synthesis of methionine, threonine, and lysine (Sirovaya et al. 2014). Also, a significant content of L-alanine (849.1 mg/100 g), Glycine (830.1 mg/100 g), L-serine (779.2 mg/100 g), L-leucine (766.9 mg/100 g), L-arginine (735.2 mg/100 g), L-proline (667.2 mg/100 g), L-threonine (660.8 mg/100 g) bound amino acids was found. L-alanine contributes as an intermediary between protein catabolism and carbohydrates synthesis. Alanine plays a central role in the metabolism of muscle protein and is the main criterion in nitrogen metabolism. With help of this acid, muscles use the energy produced by other amino acids. It is used as a component in infusion solutions, a food additive, and a precursor for pharmaceutical and chemical products (VKM 2017; Hatazawa et al. 2018; Tomé 2018). Glycine is the nonessential amino acid, which is a substrate for the synthesis of several biologically important compounds and biomolecules. It participates in detoxification reactions, in the synthesis of tripeptide

glutathione, and of proteins. Glycine has a wide spectrum of immunomodulatory, anti-inflammatory, and cytoprotective properties. To exert its effects, this acid binds to various receptors (Pérez-Torres et al. 2017). L-serine plays a major role in neurological development, cellular proliferation, brain development, and function (Koning et al. 2003). It has different functions and roles from primary protein structure to cell signaling. Deficits in this acid may have profound health effects from embryo to geriatrics. Dietary supplementation with serine is considered a probable therapy for progressive neurodegenerative diseases namely Alzheimer's diseases and myotrophic lateral sclerosis (Metcalf et al. 2018). L-leucine promotes to energy metabolism renderenergy for protein synthesis, inhibiting protein degradation (Duan et al. 2016). It plays an exceptional signaling role in adipose tissue and skeletal muscle (Li et al. 2011; Carpentier 2020), mammary epithelial cells (Lei et al. 2012), placental cells, and as well as other cell views (Kim et al. 2007; Li et al. 2007; Kim et al. 2011). L-arginine is a semi-essential amino acid, which constitutes 5–7% of the total amino acids in people with normal nutrition. This amino acid is used by all cells and by the body for protein synthesis, tissue repairing, and immune cell function. It is also converted to citrulline and act as a vasodilator (Pahlavani et al. 2014). Because of arginine's NO-stimulating effects, it can be utilized for congestive heart failure, preeclampsia, hypertension, angina pectoris, coronary heart disease, and erectile dysfunction (Appleton 2002). L-proline is an exceptional amino acid for maintaining cell structure and function (Hu et al. 2008; Kaul et al. 2008). They constitute one-third of amino acids in the collagen proteins which appertain about 30% of body proteins (Wu et al. 2011). It is the main component of collagen and promotes the healing of wounds, burns, ulcers, and good joint function. Proline contributes to buffering cellular redox potential under stress conditions, stabilizing sub-cellular structures, and scavenging free radicals (Serraj and Sinclair 2002; Hayat et al. 2012; Slobodianiuk et al. 2021b). L-threonine has been broadly used

in food and pharmaceutical manufacturing. It protects the liver by preventing the accumulation of fat in the cells and has the ability to remove toxins (Dong et al. 2012; Liu et al. 2015; Wang et al. 2019). L-cystine and L-cysteine were found only bound.

The leaves of *A. sylvestris* L. contained nineteen free amino acids and as many bound amino acids. L-glutamine, L-asparagine, and L-proline dominate among free amino acids in the studied plant these content was 547.8 mg/100 g, 377.6 mg/100 g, and 148.1 mg/100 g, respectively. Asparagine supports the normal functioning of the nervous system and liver, able to bind ammonia in tissues. Also, this acid prevents both excessive excitation and excessive inhibition, participates in metabolic processes and stimulates immunity (Sirovaya et al. 2014). Glutamine is responsible for the state of the immune system and is the main source of energy for the kidneys and intestines (Fedosov 2017). Among bound amino acids, L-glutamic and L-aspartic acids were present in the leaves of *A. sylvestris* in the greatest amount, which was 1155.6 mg/100 g, and 861.0 mg/100 g, respectively. L-cystine was found only bound.

Conclusions

By HPLC method in the leaves of *A. archangelica* L. and *A. sylvestris* L. were identified amino acids and determined their quantitative content. As a result of this research there was found in study species a significant amount of free and bound amino acids. High concentrations of free and bound amino acids such as L-glutamic acid and L-aspartic acid predominate in *A. archangelica* L. and *A. sylvestris* L. This allowed these amino acids to be considered distinguishing markers of the study plants. Character metabolic processes in which these amino acids take part may be associated with the medicinal properties of these plants pursuant to their use in medicine and, therefore, may contribute to the insight of their therapeutic properties.

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